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Cell lines that support replication of a novel herpes simplex virus 1 U_L31 deletion mutant can properly target U_L34 protein to the nuclear rim in the absence of U_L31

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Abstract

Previous results indicated that the herpes simplex virus 1 (HSV-1) U_L31 gene is necessary and sufficient for localization of the U_L34 protein exclusively to the nuclear membrane of infected Hep2 cells. In the current studies, a bacterial artificial chromosome containing the entire HSV-1 strain F genome was used to construct a recombinant viral genome in which a gene encoding kanamycin resistance was inserted in place of 262 codons of the 306 codon U_L31 open reading frame. The deletion virus produced virus titers approximately 10- to 50-fold lower in rabbit skin cells, more than 2000-fold lower in Vero cells, and more than 1500-fold lower in CV1 cells, compared to a virus bearing a restored U_L31 gene. The replication of the U_L31 deletion virus was restored on U_L31-complementing cell lines derived either from rabbit skin cells or CV1 cells. Confocal microscopy indicated that the majority of U_L34 protein localized aberrantly in the cytoplasm and nucleoplasm of Vero cells and CV1 cells, whereas U_L34 protein localized at the nuclear membrane in rabbit skin cells, and U_L31 complementing CV1 cells infected with the U_L31 deletion virus. We conclude that rabbit skin cells encode a function that allows proper localization of U_L34 protein to the nuclear membrane. We speculate that this function partially complements that of U_L31 and may explain why U_L31 is less critical for replication in rabbit skin cells as opposed to Vero and CV1 cells.

Keywords: Herpes simplex virus -1 (HSV-1); BAC; U_L31; Viral egress; U_L34; Nuclear membrane

Introduction

Herpesvirus nucleocapsids form in the nuclei of infected cells and subsequently bud from the inner nuclear membrane into the perinuclear space in a process termed primary envelopment (Mettenleiter, 2002). The mechanism of primary envelopment is unknown, but the U_L31 and U_L34 proteins have been shown to greatly facilitate the process (Chang et al., 1997; Fuchs et al., 2002; Reynolds et al., 2001; Roller et al., 2000). The U_L34 protein is essential for viral replication (Roller et al., 2000). The protein is believed to be a type II integral membrane protein with the bulk localized within the nucleoplasm in a position to interact with the U_L31 protein that also interacts with components of the nuclear lamina that line the nucleoplasmic surface of the inner nuclear membrane (Reynolds et al., 2001, 2004; Shiba et al., 2000; Yamauchi et al., 2001). The U_L31 and U_L34 proteins interact directly, colocalize at the nuclear rim of infected cells, and become incorporated into virions during envelopment at the inner nuclear membrane (Dal Monte et al., 2002; Reynolds et al., 2001, 2002; Shiba et al., 2000; Ye and Roizman, 2000; Zhu et al., 1999). In Hep2 and Vero cells, the deletion of either protein causes the other to localize aberrantly. Thus, in the absence of U_L34 protein, U_L31 localizes within the nucleoplasm, and in the absence of U_L31 protein, U_L34 protein

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localizes at the nuclear rim, in the nucleoplasm, and throughout the cytoplasm in a pattern reminiscent of the distribution of the endoplasmic reticulum (Reynolds et al., 2001, 2002).

Previously, a U_L31 null virus derived from a series of overlapping cosmids was shown to produce peak yields of infectious virus that were reduced 10,000-fold in Vero cells, and approximately 1000-fold in rabbit skin cells (Chang et al., 1997). To investigate the apparent difference in the ability of the virus to replicate in Vero and rabbit skin cells, we took advantage of a bacterial artificial chromosome (BAC) bearing the entire HSV-1(F) genome to construct a new recombinant virus bearing a deletion in U_L31. In addition, two new cell lines that expressed U_L31 were constructed. These cell lines were based on rabbit skin cells and Vero-like CV1 cells, and allowed propagation of the U_L31 deletion virus.

We have determined that propagation in Vero cells requires U_L31 protein whereas replication occurs in the absence of U_L31 in rabbit skin cells, albeit at levels reduced 10- to 50-fold relative to wild type viruses. In addition, the U_L34 protein localizes at the nuclear rim of rabbit skin cells but not Vero cells infected with the U_L31 deletion virus. This suggests that one function of U_L31, that of mediating proper localization of U_L34 protein, is mediated by a host cell function in rabbit skin cells. This observation may partly explain the ability of the U_L31 deletion virus to replicate in the rabbit skin cell line.

Results

Construction of U_L31 deletion virus v3161 and U_L31 repair virus v3161Rep

The progenitor of recombinant viruses was an HSV-1(F) genome cloned into an *Escherichia coli* bacterial artificial chromosome (BAC) designated YeBAC102 that has been previously described (Tanaka et al., 2003). YeBAC102 contains BAC vector sequences inserted into a *Bam*HI site that defines the ends of the *Bam*HI C and E fragments and is located between the U_L3 and U_L4 genes (McGeoch et al., 1985, 1986, 1988).

The basic strategy for production of a U_L31 deletion virus is outlined in Fig. 1. A gene encoding kanamycin resistance that was flanked by U_L31 sequences was amplified by polymerase chain reaction (PCR) using primers as described in Materials and methods. Sequences within the amplicon were arranged such that codons 8 to 269 of U_L31 were replaced by the gene encoding kanamycin resistance (Fig. 1). The PCR amplicon was then electroporated into the DH10B strain of *E. coli* containing (i) the

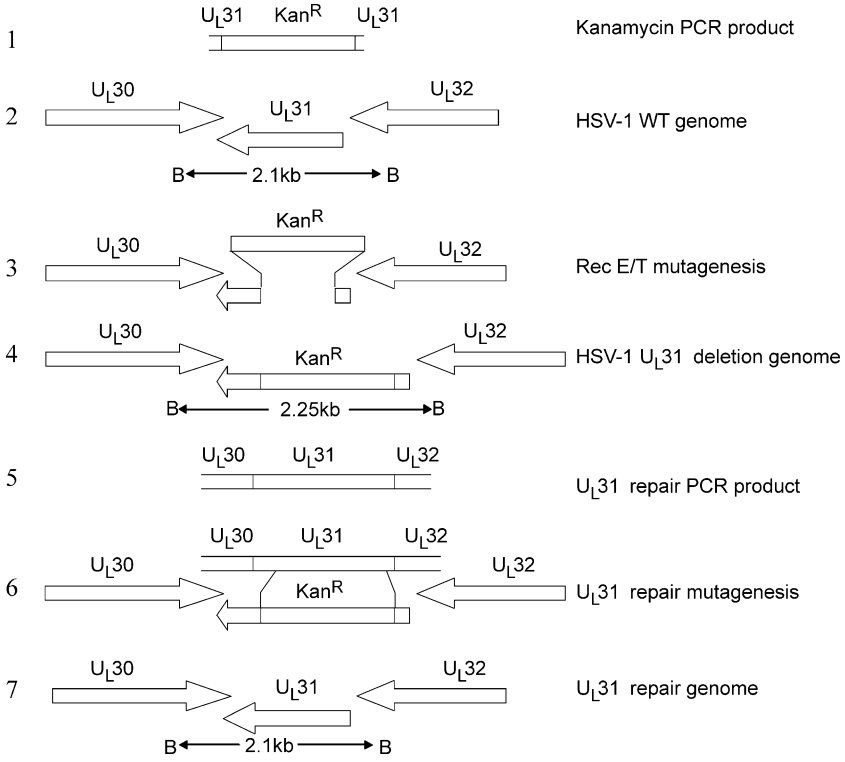


Fig. 1. Schematic diagram of steps in the construction of U_L31 deletion viruses and repair viruses. Lines 1–4: using Rec E/T-mediated recombination, a PCR amplicon bearing a kanamycin resistance cassette flanked by 50 bp of U_L31 sequences was inserted into a truncated U_L31 gene contained within an HSV-1 genome maintained in an *E. coli* artificial chromosome. Before analysis of the resulting virus, BAC sequences were removed by Cre recombinase (not shown). Lines 5–7: clone 7 rabbit skin cells (bearing U_L31) were cotransfected with a PCR amplicon bearing U_L31 and flanked by 800–1100 bp of U_L30 and U_L32, respectively, U_L31 deletion BAC DNA, and a plasmid-expressing Cre recombinase to remove BAC sequences. The resulting virus was selected on Vero cells and contained a restored U_L31 open reading frame. B, *Bam*HI.

entire HSV-1(F) genome within a bacterial artificial chromosome (pYEbac102), and (ii) a plasmid (pGETrec) encoding the Rec E/T recombination system (Narayanan et al., 1999; Tanaka et al., 2003). Colonies were plated on LB agar plates containing chloramphenicol (Camp) and kanamycin (Kan) to select for bacteria containing the BAC and the insertion into truncated U_L31, respectively.

BAC DNA was purified from individual colonies of $Camp^R$ Kan^R-bacteria and was designated BAC316. Wild type pYEbac102 DNA and BAC316 DNAs were digested with *Bam*HI, transferred to nitrocellulose, and probed with U_L31 sequences (Fig. 2A) or BAC vector DNA sequences (Fig. 2B). The U_L31 open reading within wild type BAC DNA and HSV-1(F) viral DNA is located within the 2.1 kbp *Bam*HI "W" fragment (Fig. 2A, lane 1) (McGeoch et al., 1988; Perry and McGeoch, 1988). In digests of BAC316 DNA, however, the 2.1-kbp fragment was not present, whereas a novel DNA fragment of 2.2–2.3 kbp hybridized with the U_L31-containing probe (Fig. 2, lane 2) and kanamycin probe (not shown). This size was similar to that of the expected *Bam*HI W fragment if the gene encoding Kan^R were inserted into a U_L31 gene lacking codons 8-269. Further analyses of ethidium-stained *Bam*HI digests did not reveal additional restriction polymorphisms between the wild type HSV-BAC and BAC316 (not shown). We therefore deduce that BAC316 DNA contained the kanamycin resistance marker within a truncated U_L31 open reading frame as designed.

The next step was taken to remove BAC vector sequences from BAC316 DNA and this was facilitated by the presence of *loxP* sites flanking the vector (Tanaka et al., 2003). To remove the vector sequences, BAC316 DNA was cotransfected with a Cre expression plasmid into clone 7 cells containing the U_L31 open reading fame. Viral plaques arising from the cotransfection were plaque purified twice and the resulting virus was designated v3161. To determine whether the BAC sequences were removed as a consequence of Cre activity, viral BAC316 DNA and v3161 DNA were purified and digested with *Bam*HI. The DNA was then electrophoretically separated, transferred to nitrocellulose, and probed with BAC vector sequences. As shown in Fig. 2B, a band of approximately 16 kbp hybridized with the BAC sequences in BAC316 DNA. This band is similar in size to a *Bam*HI fragment detected in pYEbac102 due to fusion of BAC sequences and the *Bam*HI C fragment (Tanaka et al., 2003). In contrast, no BAC sequences were detected in purified v3161 DNA (Fig. 2B, lane 3). Ethidium-stained *Bam*HI digests also showed a smaller *Bam*HI C fragment in v3161 compared to BAC316 and HSV-BAC due to BAC excision (not shown). We conclude that the BAC vector sequences were largely removed from the viral genome of v3161.

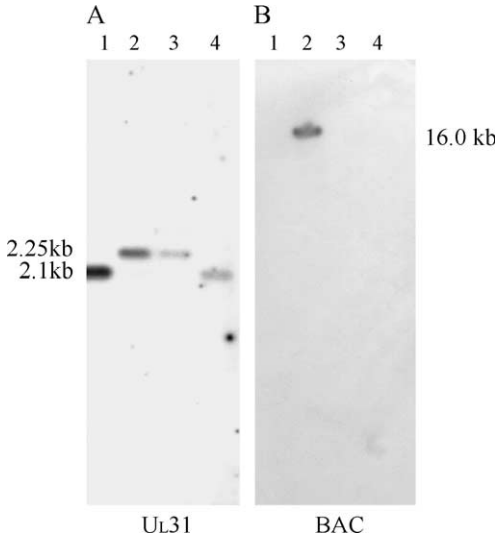


Fig. 2. Digital scan of autoradiograms of genomic DNAs from the U_L31 deletion and repaired viruses. Viral DNA of HSV-1 (F) (lane 1), U_L31 deletion-BAC (BAC316) (lane 2), U_L31 deletion virus v3161 (lane 3), and U_L31 repair viruses (v3161 Rep) (lanes 4) were digested with *Bam*HI and electrophoretically separated on a 1% agarose gel, and transferred to two nitrocellulous membranes. The membranes were then probed with [^{32}P]-labeled U_L31 sequences (panel A) or [^{32}P]-labeled BAC vector sequences (panel B).

To ensure that any phenotype of v3161 could be ascribed to the insertion/deletion of U_L31, a virus derived from v3161 was generated that bore a restored U_L31 gene. Thus, DNA that contained U_L31 and approximately 0.8 kbp of flanking sequences on one side and 1.1 kbp on the other was generated by PCR. The PCR amplicon was then cotransfected with BAC316 DNA and the Cre expression plasmid into U_L31-rescuing clone 7 cells. Progeny virus from the cotransfection was then plated on Vero cells and virus from well isolated viral plaques were plaque purified two additional times. One of these viruses was designated v3161Rep and was used for further studies. As shown in Fig. 2, the *Bam*HI W fragment of v3161Rep was restored to a size indistinguishable from that of the wild type HSV-1 BAC DNA. Moreover, v3161Rep did not hybridize with the BAC vector sequences, indicating that the Cre recombinase removed the vector sequences as a consequence of recombination mediated by the sequences flanking BAC vector sequences in YeBAC102 (Fig. 2B, lane 4).

Growth phenotype of U_L31 deletion v3161 and repair virus v3161Rep on different cell lines

As a first step to characterize the growth phenotype of the U_L31 deletion virus, the U_L31-containing cell line clone 7, rabbit skin cells, and Vero cells were infected with U_L31 deletion virus v3161 at a multiplicity of infection (MOI) of approximately 0.001 PFU per cell. At 2 days after infection, plaques were readily observed on clone 7 cells (Fig. 3). The plaques grew markedly in diameter on day 3 and afterwards. Surprisingly, small plaques were also observed on rabbit skin cells not expressing U_L31; these plaques grew slowly in diameter over several days, but never reached the size of plaques seen on clone 7 cells. In contrast, Vero cells contained focal areas of cytopathic effect that involved

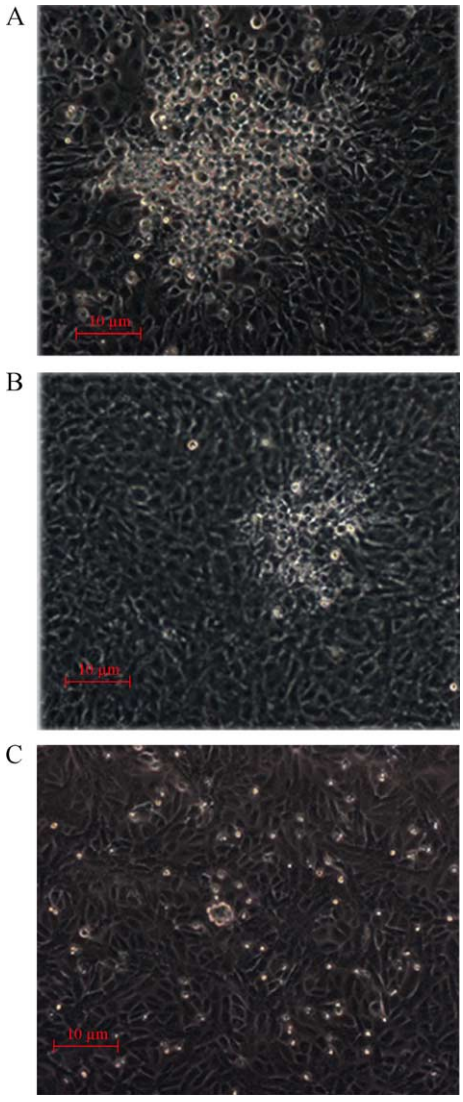


Fig. 3. Plaque phenotypes of U_L31 deletion virus on different cell types. Monolayers of rescuing clone 7 cells that were derived from rabbit skin cells (panel A), rabbit skin cells (panel B), and Vero cells (panel C) were infected with U_L31 deletion virus v3161 at low MOI (0.001). At 45 h, P.I., phase contrast photographs were recorded. The length of the bar represents 10 micrometers.

approximately 2–8 cells. These very tiny plaques did not increase markedly in diameter with time. However, the plaque sizes of the repair virus v3161Rep were indistinguishable on all three cell lines (not shown).

To further examine the effect of U_L31 on the growth of HSV in different cell types, the rescuing clone 7 cells, rabbit skin cells, Vero cells and human 143 cells in 25 cm^2 dishes were infected with v3161 or v3161Rep at an MOI of 0.1. The cells were washed extensively with 199V medium, and were subsequently harvested at 0, 6, 12, 18, and 24 h after attachment. The amount of infectious virus in each flask was then titered on clone 7 cells. As shown in Fig. 4, the U_L31 deletion virus v3161 grew to titers of approximately 2×10^6 PFU per ml in the rescuing clone 7 cells, and to approximately 10- to 50-fold lower titers in rabbit skin cells (panel A) and 143 cells (panel A). The eclipse phase of the growth curve was pronounced in these experiments, and was extended to longer than 12 h in 143 cells.

The U_L31 deletion virus was unable to replicate appreciably on Vero cells infected at an MOI of 0.1, and titers remained near that of the inoculated input virus (panel A). Similar results were obtained with Hep2 cells (not shown). In contrast, the virus v3161Rep, bearing a restored U_L31 gene, grew to comparably high titers on all of the cell lines tested (panels B and D). The eclipse phase in Vero cells infected with v3161Rep at an MOI of 0.1 was prolonged relative to rabbit skin cells and clone 7 cells.

To further test whether the U_L31 deletion mutant was able to replicate to a limited extent in Vero cells, similar growth experiments were performed at higher MOIs. Thus, clone 7 cells, rabbit skin cells, and Vero cells were infected with v3161 and v3161Rep at an MOI of 1.0, and washed with citrate buffer (pH 3.0) after attachment to remove as much input virus as possible. At various times later, cells were lysed and viruses were titered on the complementing cell line clone 7 (panel C and D). Vero cells were severely debilitated in their ability to support growth of the U_L31 deletion virus as opposed to the clone 7 complementing cell line. Nevertheless, the amount of U_L31 deletion virus increased about 10- to 30-fold on Vero cells (panel C) after a period of 24 h. We conclude that although the U_L31 gene is ultimately nonessential for viral growth in all cell lines tested, replication in Vero cells was highly dependent on U_L31 whereas replication in rabbit skin cells was at least 100-fold less dependent.

Localization of U_L34 protein in infected RSC and Vero cells

Functions of U_L31 include an important role in nucleocapsid envelopment and a minor one in DNA packaging (Chang et al., 1997; Reynolds et al., 2001, 2002). Complicating these interpretations, however, is the observation that the U_L31 protein is also essential for proper targeting of U_L34 protein to the nuclear membrane; thus, any of the putative roles ascribed to U_L31 may be a consequence of the effects on U_L34 and vice versa.

To determine if proper targeting of U_L34 protein by U_L31 correlated with the ability of the U_L31 deletion virus to replicate in various cell lines, Vero cells, rabbit skin cells, and the complementing clone 7 cells were infected with the U_L31 deletion virus, and 16 h later, the cells were fixed and immunostained for the presence of U_L34 protein. As shown previously in Hep2 cells (Reynolds et al., 2001), U_L34 protein localized aberrantly in the cytoplasm, nucleoplasm, and nuclear rim of infected Vero cells rather than just the nuclear rim pattern of immunostained cells infected with wild type HSV-1(F) (Fig. 5B). In contrast to these results, U_L34 protein localized at the nuclear rim of rabbit skin cells (Fig. 5A) and clone 7 cells (not shown) infected with the

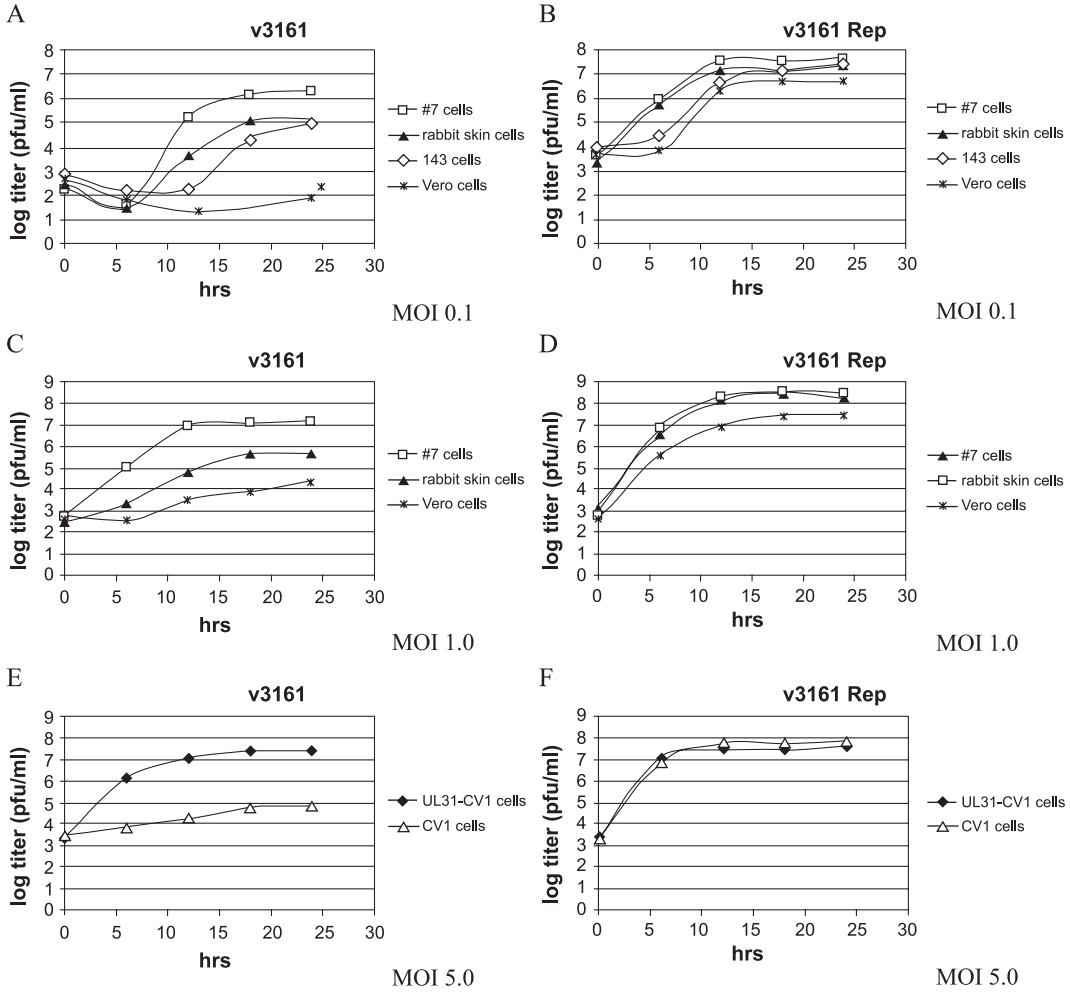
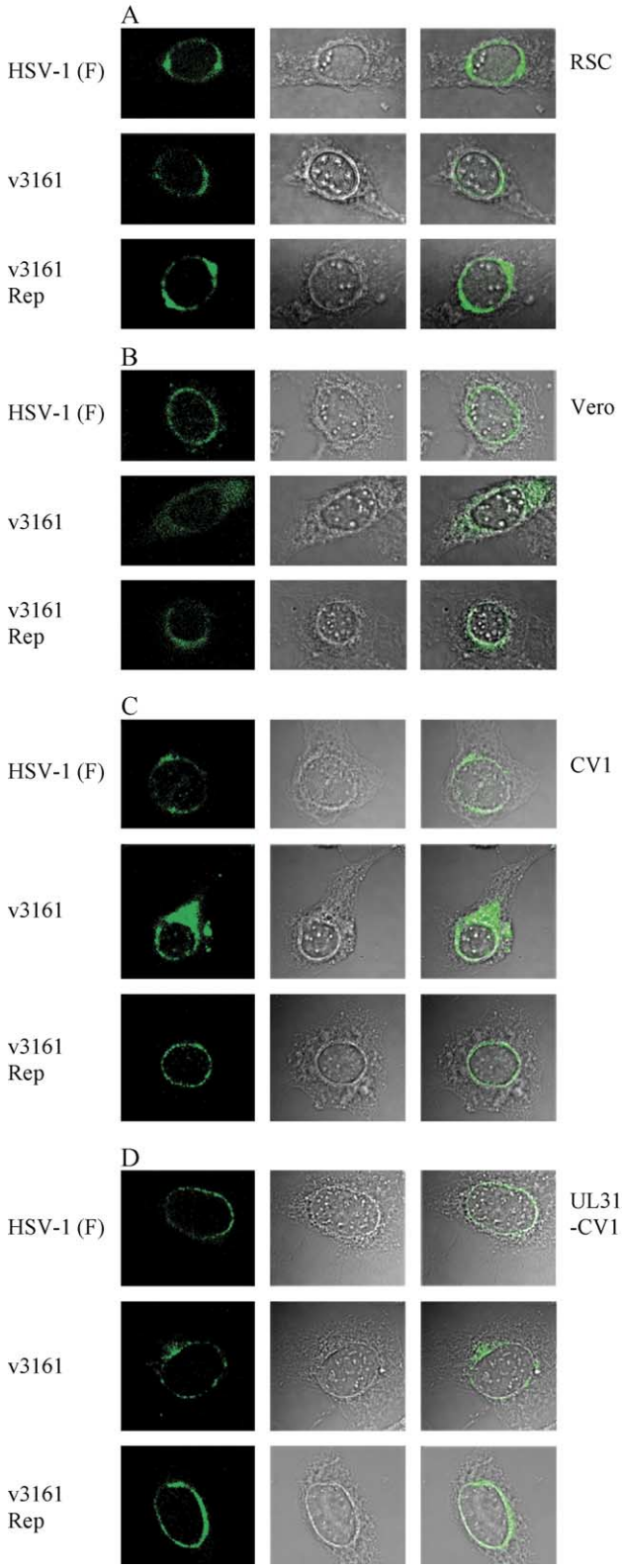


Fig. 4. Growth curves of U_L31 deletion viruses and repair viruses in various cell lines. Monolayers of rabbit skin cells, clone 7 cells, 143 cells, and Vero cells in 25 cm^2 dishes were infected with U_L31 deletion v3161 or repaired virus v3161Rep at an MOI of 0.1 (panel A, B) or 1.0 (panel C, D). The cells were either washed without (panel A, B) or with low pH citrate buffer to remove extracellular virus (panel C, D). Cells were harvested at 0, 6, 12, 18, and 24 h post-infection, and virus in cell lysates was titrated on clone 7 cells. CV1 cells and U_L31-containing CV1 cells were infected with U_L31 deletion v3161 (panel E) or repair virus v3161Rep (panel F) at MOI 5, washed with citrate buffer, and harvested at 0, 6, 12, 18, and 24 h P.I. Viral stocks were titrated on U_L31-CV1 cells.

U_L31 deletion virus. A similar nuclear rim appearance was noted in all cells types infected with the U_L31 repair virus (Fig. 5). We therefore conclude that proper localization of U_L34 protein in Vero cells requires U_L31 protein, but that rabbit skin cells encode a function that complements this function of U_L31 and partially restores replication of the U_L31 deletion mutant.

The next set of experiments was designed to determine if U_L31 was sufficient to convert a cell that was normally nonpermissive for U_L31 deletion virus replication to a permissive cell phenotype, and then to determine if this conversion correlated with proper localization of U_L34 protein. We chose CV1 cells harboring a *Frt* target sequence for these experiments because a system to rapidly generate recombinant cell lines using Flp recombinase to drive genes of interest into the Frt locus was commercially available. A transfer plasmid carrying U_L31 driven by the cytomegalovirus promoter and a hygromycin resistance cassette flanked by *Frt* sites was cotransfected into the CV1 cell line with a plasmid encoding the Flp recombinase. Hygromycin-resistant cell clones were then propagated that presumably expressed U_L31 protein. The parent CV1 cells did not support plaque formation of the U_L31 deletion virus (not shown), nor viral replication of the U_L31 deletion virus (Fig. 4E). Specifically, replication of the U_L31 deletion virus was limited to an approximately 20-fold increase above input levels over a 24-h period, even when infected at an MOI of 5.0 (Fig. 4E). Moreover, the localization of U_L34 protein in CV1 cells was aberrant. The U_L34 protein-specific immunostaining remained largely in the cytoplasm, with less in the nucleoplasm and only a limited amount located at the nuclear rim (Fig. 5C). In contrast, CV1 cells engineered to express U_L31 fully restored replication of the U_L31 deletion virus such that it was indistinguishable from that of wild type virus and a virus bearing a restored U_L31 gene (Fig. 4E). In addition, the nuclear rim localization of U_L34 protein was restored in the engineered cell line (Fig. 5D). We therefore conclude that expression of U_L31 protein is

sufficient to convert a cell line that does not normally support replication of a U_L31 deletion virus to one that is permissive. Importantly, this conversion correlated with proper localization of U_L34 protein with which the U_L31 protein interacts.



Discussion

These studies expand upon previous work using a U_L31 deletion virus derived from a cosmid reconstitution system (Chang et al., 1997). Specifically, this work demonstrates marked differences in the abilities of various cell lines to support replication and plaque formation of a U_L31 deletion virus. The permissiveness of a given cell line correlated strongly with the capacity of the cell line to mediate proper localization of the U_L34 protein to the nuclear rim.

In a previous study, peak titers of a U_L31 deletion mutant were reduced approximately 1000-fold in rabbit skin cells and 10,000-fold in Vero cells when compared to titers produced by wild type viruses at about 26 h of infection. This is the first study to perform growth curves of U_L31 deletion viruses in various cell lines. Growth curves were performed after eliminating input virus before the onset of viral replication to document very limited viral replication over time in various cell lines. Infection with the U_L31 deletion produced a 50-fold increase in infectious titer in Vero cells over 24 h, over a 1000-fold increase in rabbit skin cells, and more than 10,000-fold in cells engineered to express U_L31. Replication in rabbit skin cells was reduced 20- to 200-fold (depending on the time point and amount of input virus) compared to a virus derived from the U_L31 deletion virus that contained a restored U_L31 gene. In addition, this is the first study to investigate plaque formation in the various cell lines. The fact that U_L31 protein augmented, but was ultimately dispensable for, cell to cell spread in rabbit skin cells was supported by the observation that small plaques formed on normal rabbit skin cells infected with the U_L31 deletion virus. In contrast, only groups of 4–8 rounded Vero cells were observed after infection with the U_L31 deletion virus.

One reason that peak titers in the current studies were higher in rabbit skin cells than in the previous study may stem from the fact that the current study used a different cell line (clone 7) as an indicator in the titration assays. This cell line was derived from rabbit skin cells that were already partially able to complement the U_L31 deletion virus. Moreover, clone 7 cells were highly permissive for replication of all viruses tested (including wild type viruses) when compared to either Vero or rabbit skin cells. Given the highly permissive nature of the indicator cell line, it would be expected that the current study may represent a very sensitive detection of infectious virus. Supporting the

Fig. 5. Confocal microscopic images of HSV-1 infected cells immunostained for U_L34 protein. Rabbit skin cells (panel A), Vero cells (panel B), CV1 cells (panel C), or CV1 engineered to contain U_L31 (panel D) were infected with HSV-1 (F), U_L31 deletion virus v3161 or U_L31 repair virus v3161Rep for 16 h and then fixed with ice-cold methanol. Cells were then immunostained with U_L34 protein-specific chicken antibody, and the immunofluorescence was detected by FITC-conjugated donkey anti-chicken IgY antibody. Differential interference contrast and fluorescence in each field were recorded separately and these were merged in a separate image.

possibility that the different cell lines contributed at least partly to differences in results of the two studies is the observation that the previously described U_L31 deletion virus (Chang et al., 1997) yielded higher infectious titers on clone 7 cells than on the previously described U_L31-expressing cell line (data not shown).

The mechanism to explain why U_L31 protein is essential for viral replication on Vero and Hep2 cells, but not rabbit skin or 143 cells may partly be explained by the observation that U_L34 protein was correctly localized to the nuclear rim in rabbit skin cells (Fig. 5A), and human 143 cells (not shown), but aberrantly localized in human Hep2 cells (Reynolds et al., 2001) and Vero cells (Fig. 5B). Thus, at least one function of U_L31 (that of proper targeting of U_L34 protein) is complemented in the more permissive cell lines. This suggests that U_L34 protein interacts with cellular proteins and indicates that in some types of cells, U_L31 is not necessary to mediate proper U_L34 protein targeting to the nuclear rim. Alternatively, the permissivity of rabbit skin cells may be only partly explained by the proper targeting of U_L34 protein. Inasmuch as U_L31 likely serves to modify the nuclear lamina and thereby promote nucleocapsid egress (Reynolds et al., 2004), it is possible that rabbit skin cells have a more "porous" lamina that does not require U_L31-mediated disruption or thinning to allow nucleocapsids access to the inner nuclear membrane. Further studies will be necessary to address the nature of the lamina in different cell types.

Materials and methods

Plasmids

HSV-BAC (pYEbac102) was maintained in the DH10B strain of *E. coli* under chloramphenicol selection as previously described (Tanaka et al., 2003). The plasmid pCAGGS-nlsCre plasmid was a gift from Dr. Michael Kotlikoff, Cornell University. The True Blue Bac II plasmid was obtained from Genomics One Corporation. Plasmid pJB233 contains U_L31 open reading frame in pCDNA3 (Invitrogen), and pJB353 contains the U_L31 open reading frame in pCDNA5/FRT (Invitrogen) harboring a Flp recombinase targeting site.

Cells and viruses

Rabbit skin cells and Vero cells were maintained in DMEM containing 10% newborn calf serum, penicillin and streptomycin; human 143(TK-) cells were maintained in DMEM containing 10% fetal bovine serum with penicillin and streptomycin. To construct a U_L31-expressing cell line procedures similar to those described previously were employed (Reynolds et al., 2001). Briefly, the U_L31 open reading frame was cloned into the vector plasmid pCDNA3 (Invitrogen). The final plasmid contained U_L31 under the control of the human cytomegalovirus immediate early promoter/enhancer and included a gene encoding neomycin resistance. G418-resistant cells were selected in 200 μg per ml G418 in DMEM supplemented with 10% newborn calf serum, and were cloned once by limiting dilution before they were tested for the ability to support replication of the U_L31 deletion virus. One clone that supported replication of the U_L31 deletion virus was selected for further studies and was designated clone 7.

Another U_L31 complementing cell line was constructed using the Flp-In-CV1 system (Invitrogen) according to the manufacturer's protocol. The U_L31 open reading frame was cloned into the pCDNA5/FRT vector bearing a Flp recombinase targeting site, and this new plasmid was designated as pJB353. pJB353 was then cotransfected into Flp-CV1 cells with the pOG44 plasmid-expressing Flp recombinase. Stably transfected cells were selected under 200 μg/ml hygromycin B and tested for the ability to rescue the U_L31 deletion virus. The final cell line was designated as U_L31-CV1 cells.

Construction of an HSV-1(F) BAC bearing a deletion in U_L31

The DH10B strain containing the HSV-1(F) BAC plasmid and a previously described plasmid, pGET-rec encoding the Rec E/T recombinase under the control of an arabinose-inducible promoter (a kind gift of Dr. P.A. Ioannou, Murdoch Institute for Research into Birth defects) was plated on LB agar containing 30 μg/ml chloramphenicol and 50 μg/ml ampicillin (Narayanan et al., 1999). A single colony was picked and grown in 5 ml LB medium overnight. Seventy ml of LB medium without glucose was inoculated with 0.7 ml of overnight culture and was incubated at 37 °C in a bacterial shaker. Once the culture reached an OD600 of 0.1–0.15, 0.7 ml 10% L-arabinose was added to induce expression of the ET recombinase. After further incubation at 37°, the cells were harvested at an OD600 of 0.4. Cells were pelleted for 10 min at 8000 × *g* at 4 °C, the supernatant was decanted, and the flask was placed on ice. The pelleted bacteria were resuspended in 10 ml ice cold 10% glycerol (pre cooled on ice at least 3 h before use) using an ice cold pipette. A further 50 ml of ice cold 10% glycerol was added to the tube and the bacteria were again pelleted. This step was repeated twice. The supernatant was again decanted. The inside of the tube was blotted with absorbent paper, and the cells were resuspended in the remaining 100 μl of liquid. Fifty microliters of cells were transferred into precooled eppendorf tubes that were stored briefly on ice before electroporation.

An amplicon containing a gene encoding kanamycin resistance and 50 bp of flanking U_L31 sequences on each side was generated using the primers: 5′CTC GCT CCT GTC CCT GGA GCA CAC CCT GTG TAC CTA TGT ATG ACA CCG ACC CCC ATC GAC AGC AAG CGA ACC GGA ATT GCC AGC3′ and 5′CAT GAG CCC CCC

GTC GAA GCT GAT GTC CCT CAT TTT ACA ATA AAT GTC TGC GGC CGA CAC GGA AAT GTT GAA TAC TCA TAC TCT TCC TTT TTC AAT TCA G3′ using pCR2.1 plasmid (Invitrogen) as template. The primers were designed to replace codons 8 to 269 of U_L31 with a kanamycin resistance gene. Approximately 1 μg of PCR product in a maximum volume of 5 μl was mixed with 50 μl of the electrocompetent bacteria in an ice-cold cuvette and electroporated at 2.3 kV in a Bio-Rad Gene Pulser, at 25 uF with the pulse controller set to 200 Ω. One milliliter of LB medium was added to resuspend the bacteria, and the material was transferred back to an eppendorf tube and incubated at 37 °C for 1 h with shaking.

Bacteria from the electroporation were then plated on LB agar plates containing 50 μg/ml kanamycin and 30 μg/ml chloramphenicol to select bacteria containing the kanamycin resistance gene inserted into the U_L31 locus. BAC DNA from various colonies was purified using standard alkaline lysis methods for plasmid purification (Kirschner and Stratakis, 1999; Sambrook et al., 1989), and the genotype was examined by restriction enzyme analyses and hybridization with appropriate radiolabeled DNA probes as indicated in the results section. The virus containing kanamycin inserted in U_L31 was designated as BAC316. Clone 7 cells were cotransfected with BAC316 DNA and a Cre expression plasmid (pCAGGS-nlsCre, a kind gift from Dr. Michael Kotlikoff, Cornell University) to remove BAC sequences from the reconstituted viral genome. The virus was designated v3161 and the expected genotype was confirmed as indicated below and in Results.

The virus bearing a restored U_L31 repair gene was constructed as follows. The U_L31 with 0.8 kbp flanking sequence on one side and 1.1 kbp on the other side was amplified by PCR with primers: 5′CGG ACT CCA TCT TTG TGC TGT GC 3′ and 5′CGG ATC TGG TCC TGT TGC TCC TC 3′. Clone 7 cells were cotransfected with the PCR product, the U_L31 deletion-BAC DNA and Cre expression plasmid. Viral plaques were selected and plaque purified twice on Vero cells. One plaque was selected for further studies and the virus therein was designated as v3161Rep.

Purification of HSV-1 viral DNA

Cells in 25 cm^2 dishes were infected at 5.0 PFU per cell with the indicated viruses until cytopathic effect was visible in 80% of the cells. The cells were then removed into cold PBS, pelleted at 2500 × *g* for 5 min, and resuspended in 0.4 ml TE (10 mM Tris–HCl, pH 8, 1 mM EDTA). De-enveloped capsids were released into solution after incubation on ice for 15 min followed by addition of 20 μl 20% Triton X-100. After clarification at 2000 × *g*, the supernatant was decanted into a 1.5-ml microfuge tube. Proteinase K (20 μl of a 10 mg/ml solution) and 40 μl 10% SDS were added. After incubation at 37 °C for 30 min, the samples were extracted with an equal volume of 49.5% phenol/49.5% chloroform and 1% isoamyl alcohol. DNA was precipitated from the aqueous phase at −20 °C overnight in 0.1 volume 3 M sodium acetate (pH 5.5) and 2.5 volumes ethanol, followed by centrifugation at 4 °C for 10 min. DNA in the dried pellets was then resuspended in water for subsequent restriction enzyme digestion.

Hybridization of viral DNA

Viral DNA was purified as described above and digested with *Bam*HI. DNA was then separated on a 1% agarose gel. The gel was soaked twice in two volumes of 0.25 M HCl for 15 min with gentle agitation, and then soaked twice in two volumes of denaturation solution (0.5 N NaOH and 1.5 N NaCl) for 15 min with agitation. The gel was then soaked twice in neutralization buffer (1 M Tris–HCl and 2.0 M NaCl) for at least 30 min each time. Viral DNA within the gel was then transferred to a nitrocellulous membrane and crosslinked to the membrane by exposure to UV light. A DNA probe was labeled with $[\alpha\text{-dCTP}]^{32}P$ (Amersham) using a nick translation kit (Gibco-BRL) and precipitated with single stranded Herring sperm DNA and 95% ethanol to remove free $[\alpha dCTP]^{32}P$. The probe was then denatured by boiling for 15 min and rapid cooling on ice. The nitrocellulous membrane was then incubated with hybridization buffer which consists of 4 × SSC (Sambrook et al., 1989), 100 μg/ml denatured single strand sperm DNA, 30% Formamide, 1 × Denhardt's solution and 0.1% SDS at 65 °C for 10 min. Denatured probe was then added and incubated overnight at 65 °C. The next day, the blot was washed at 42 °C twice for 5 min with 2 × SSC, 0.1% SDS, and for 15 min with 0.4 × SSC, 0.1% SDS at least three times at 37 °C. The blot was then exposed to X-Omat ray film at −80 °C, followed by film development.

Single-step growth curve experiments

Rabbit skin cells, a derivative of rabbit skin cells bearing U_L31 (clone 7), 143 cells, Vero cells, CV1 cells and CV1 cells bearing U_L31 (U_L31-CV1) cells in 25 cm^2 dishes were infected with the U_L31 deletion virus v3161 or U_L31 repair virus v3161Rep in medium 199 supplemented with 1% newborn calf serum (199V). Virus was allowed to adsorb to the cells for 1 h at 37 °C with agitation. Cells were washed once with 199V supplemented with human serum containing neutralizing antibody to HSV-1, and three times with 199V or two times with CBS buffer (40 mM citric acid, 10 mM KCl, 135 mM NaCl, pH 3.0). This point was designated time 0. Five-milliliter growth media was added to the infected 143 cells, whereas 5 ml of 199 V was added for the other cell lines. Cells were incubated in a humidified chamber at 37 °C in an atmosphere of 5% CO_2, and were harvested at 0, 6, 12, 18, and 24 h of incubation. Infectious virus was then titrated on clone 7 cells. Viruses harvested from CV1 and U_L31-CV1 cells were titrated on U_L31-CV1 cells.

Light microscopy

Clone 7 cells, rabbit skin and Vero cells in 25 cm^2 dishes were infected with U_L31 deletion virus v3161 in 199 V for 45 h. Plaques were examined under the Zeiss microscope equipped with an AxioCam HR camera. Images were processed using Axiovision 3.1 software.

Confocal microscopy

Rabbit skin cells, Vero cells, CV1 cells, and U_L31-CV1 cells were seeded on glass coverslips in 10 cm^2 wells of a 6-well plate to reach 90% confluency and infected with wild type HSV-1, the U_L31 deletion virus v3161, and U_L31 repair virus v3161 Rep. At 16 h after infection, cells were fixed with ice-cold methanol for 20 min at −20 °C. Cells were then blocked in 10% BlockHen II (from Aves Lab, Inc) for an hour and stained with U_L34 anti-chicken antibody that was kindly provided by Dr. Richard Roller at 1:4000 (Roller et al., 2000). FITC conjugated secondary antibody was used at 1:100 (Immuno-Jackson). Slides were examined with an Olympus confocal laser-scanning microscope using 10× ocular and 40× stage objectives and an excitation wavelength of 488 nm. Cells were also examined by Nomarski differential interference contrast imaging as described previously (Reynolds et al., 2001).

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